

1 Concentrating mixtures of neuroactive pharmaceuticals and
2 altered neurotransmitter levels in the brain of fish exposed to a
3 wastewater effluent

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21 **Abstract**

22 Fish can be exposed to a variety of neuroactive pharmaceuticals via the effluent
23 discharges from wastewater treatment plants and concerns have arisen regarding their
24 potential impacts on fish behaviour and ecology. In this study, we investigated the uptake of
25 14 neuroactive pharmaceuticals from a treated wastewater effluent into blood plasma and
26 brain regions of roach (*Rutilus rutilus*) after exposure for 15 days. We show that a complex
27 mixture of pharmaceuticals including, 6 selective serotonin reuptake inhibitors, a serotonin-
28 noradrenaline reuptake inhibitor, 3 atypical antipsychotics, 2 tricyclic antidepressants and a
29 benzodiazepine, concentrate in different regions of the brain including the telencephalon,
30 hypothalamus, optic tectum and hindbrain of effluent-exposed fish. Pharmaceuticals, with the
31 exception of nordiazepam, were between 3-40 fold higher in brain compared with blood
32 plasma, showing these neuroactive drugs are readily uptaken, into brain tissues in fish. To
33 assess for the potential for any adverse ecotoxicological effects, the effect ratio was
34 calculated from human therapeutic plasma concentrations (HtPCs) and the measured or
35 predicted fish plasma concentrations of pharmaceuticals. After accounting for a safety factor
36 of 1000, the effect ratios indicated that fluoxetine, norfluoxetine, sertraline, and amitriptyline
37 warrant prioritisation for risk assessment studies. Furthermore, although plasma
38 concentrations of all the pharmaceuticals were between 33 to 5714-fold below HtPCs,
39 alterations in serotonin, glutamate, acetylcholine and tryptophan concentrations were
40 observed in different brain regions of effluent-exposed fish. This study highlights the
41 importance of determining the potential health effects arising from the concentration of
42 complex environmental mixtures in risk assessment studies.

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44 **Keywords.** Fish, antidepressants, antipsychotics, SSRIs, neurotransmitters, wastewater
45 effluents

1. Introduction

After human ingestion, many pharmaceuticals are excreted in their native form or as metabolites and they enter aquatic systems mainly via effluents from wastewater treatment works (WwTWs) (Fent et al., 2006; Gardner et al., 2012; Kostich et al., 2014; Petrie et al., 2015; Tanoue et al., 2015). Recently, concerns have arisen regarding the presence of neuroactive pharmaceuticals, such as selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines, in WwTW effluents because these pharmaceuticals have been shown to alter behaviour of different fish species under experimental laboratory conditions (Brodin et al., 2013; Huerta et al., 2016; Margiotta-Casaluci et al., 2014; Valenti et al., 2012). In addition to SSRIs, other types of antidepressants including serotonin-noradrenaline reuptake inhibitors (SNRIs) and tricyclic antidepressants are also regularly detected in effluents from WwTWs (Grabicova et al., 2014; Lajeunesse et al., 2011; Petrie et al., 2015). In comparison to SSRIs, the effects of tricyclic antidepressants on fish behaviour have been less studied, but a member of this class, amitriptyline, is extensively prescribed in England to treat depression and psychiatric disorders (>10,000 kg in England in 2012) (Petrie et al., 2015; Ziarrusta et al., 2017).

Recent studies investigating the effects of neuroactive pharmaceuticals on fish have focused on behavioural endpoints as these are likely to be the primary effects of exposure to neuroactive pharmaceuticals according to the read-across hypothesis (Margiotta-Casaluci et al., 2014; Valenti et al., 2012). This hypothesis has been suggested to be a useful tool in risk assessment studies and is based on the theory that blood plasma concentrations of pharmaceuticals in non-target vertebrates similar to human therapeutic plasma concentrations are likely to cause pharmacological effects in organisms such as fish (Huggett et al., 2003; Rand-Weaver et al., 2013). However, there is uncertainty as to whether environmental water

concentrations of neuroactive drugs such as SSRIs bioconcentrate to high enough concentrations in the plasma or target tissues to cause behavioural effects in fish (Sumpter et al., 2014). Mean measured concentrations of neuroactive pharmaceuticals in WwTW effluents have been reported at between 1 and 330 ng/L for SSRIs (e.g., fluoxetine, sertraline and citalopram), SNRIs (venlafaxine), tricyclic antidepressants (amitriptyline) and benzodiazepines (diazepam) (Grabicova et al., 2014; Petrie et al., 2015), and these are far below water concentrations for SSRIs fluoxetine, sertraline and citalopram (in the range of 1-116 µg/L) and for venlafaxine (between 250 and 500 µg/L) reported to affect the behaviour of different fish species in laboratory experiments (Bisesi et al., 2014; Kellner et al., 2016; Margiotta-Casaluci et al., 2014; Valenti et al., 2012; Xie et al., 2015). In contrast with these reports however, there have been some studies indicating that fluoxetine at concentrations below 1 µg/L (0.3 µg/L and 0.54 µg/L), and therefore similar to environmental concentrations, could affect fish behaviour (Barry, 2013; Dziewieczynski and Hebert, 2012). Furthermore, non-monotonic shifts in behavioural responses have been reported for neuroactive compounds such as oxazepam (Huerta et al., 2016). More information is needed on the uptake and tissue partitioning of mixtures of neuroactive pharmaceuticals in fish to support the environmental risk assessments of these drugs in aquatic environments.

In a recent study, we used a holistic untargeted chemical profiling approach to identify pharmaceuticals accumulating in roach (*Rutilus rutilus*) exposed to a treated wastewater effluent. We identified 14 different neuroactive compounds in extracts of either gonad, liver, gills, kidney or plasma (David et al., 2017). The aim of the study presented here was to use targeted analyses to quantify the concentrations of this pharmaceutical mixture in plasma and brain and investigate for biological responses in the brain via measurement of neurotransmitter concentrations as a consequence of effluent exposure. We provide novel information on the accumulation pattern of neuroactive pharmaceuticals in four different

regions of the brain, i.e. the telencephalon, hypothalamus, optic tectum and hindbrain. We show that even though plasma concentrations of all neuroactive drugs were well below the human therapeutic plasma concentrations, concentrations of neurotransmitters were altered in the hypothalamus of effluent-exposed fish, highlighting the need to understand the effects of exposure to complex chemical mixtures on fish behaviour and physiology.

2. Materials and Methods

2.1 Chemicals and reagents

Certified standards of glutamate, acetylcholine, serotonin, tryptophan, fluoxetine (Fluo), sertraline (Ser), paroxetine (Par), clozapine (Clo), venlafaxine (Venlaf), and also formic acid, ammonium hydroxide, phosphoric acid were obtained from Sigma Aldrich, Dorset, UK. Norfluoxetine (norFluo), norsesraline (norSer), citalopram (Cit), norclozapine (norClo), quetiapine (Quet), amitriptyline (Ami), noramitriptyline (norAmi), diazepam (Diaz), nordiazepam (norDaz), serotonin-d₄, norfluoxetine-d₆ were purchased from LGC standards UK. Venlafaxine-d₆, fluoxetine-d₅ were purchased from QMX Laboratories Limited UK. All standards were > 99% compound purity and deuterated standards > 97% isotopic purity. HPLC grade acetonitrile, methanol and water were obtained from Rathburns UK. Oasis HLB cartridges (1 g) were obtained from Waters, Manchester, UK and Strata-X-C Polymeric Reversed Phase 96-Well Plates (33 µm sized particles, 30 mg/well) from Phenomenex, Macclesfield UK.

2.2 Fish exposure and sample collections

A population of male and female sexually mature roach (*Rutilus rutilus*, age 2+, mean \pm SEM length of 14.5 ± 1.3 cm and weight 45.4 ± 12.1 g) was exposed for 15 days to either a treated effluent from a WwTW or to clean water for the control population (60 fish per

treatment and 120 fish in total). The WwTW received 95% influent from domestic sources (population equivalent of 117,574) and 5% input from industrial wastewaters. The influent was treated by fine screens, chemically assisted settlement, biological aerated flooded filter processes and ultraviolet disinfection. The pH of the effluent was 7.3, concentrations of suspended solids 21 mg/L, biochemical oxygen demand 11 mg/L, chemical oxygen demand 67 mg/L and total ammonia 29 mg/L during the exposure period (South West Water data). Roach were exposed in triplicate tanks (20 fish per 200 L tank) to either dechlorinated water or 100% final effluent for fifteen days. The flow rate was 10 L/min and the effluent and water was continually aerated. The fish were maintained at an average temperature of 12 ± 1 °C for both treatments under a constant photo-period (15h light : 9h dark).

After 15 days, fish were terminated using an overdose of phenoxyethanol and according to UK Home Office regulations and local ethical guidelines. Blood was collected from the caudal vein using heparinised needles (BD Microlance 3 25G 0.5 x 25 mm), centrifuged (5 min, 10,000 g) and the plasma supernatant stored at -70 °C. Four different parts of the brain, i.e. the telencephalon, the hypothalamus, the optic tectum and the hindbrain were carefully dissected and immediately snap frozen in liquid nitrogen. All samples were stored at -70 °C until analysis.

Wastewater effluent samples (2 x 2.5 litres) were collected in solvent washed, acid rinsed amber glass bottles at the beginning of the fish exposure (day 0), after 7 days, and at the end of the exposure period (day 15) in order to monitor the concentrations of neuroactive pharmaceuticals in the wastewater effluent throughout the exposure period. Samples were stored at 4°C with the addition of acetic acid (1%) and methanol (5%) and were extracted within 12 h after collection.

2.2 Sample preparation

2.2.1 Brain tissues and plasma samples

Telencephalon (mean \pm standard deviation; 18 ± 4 mg), hypothalamus (15 ± 3 mg), hindbrain (56 ± 8 mg) and optic tectum (80 ± 10 mg) tissues were extracted in glass tubes with 300 μ L of methanol and using a Microson XL2000 ultrasonic probe (Misonix Farmingdale) (10W x 40s). Before extraction, the mix of internal standards (IS) (serotonin-d4, venlafaxine-d6, fluoxetine-d5 and norfluoxetine-d6) was added to each tissue sample. The amount of IS added were adjusted to the initial mass of tissue used so that the amount of each IS corresponded to 1.5 ng/18 mg of telencephalon, 1.5 ng/15 mg of hypothalamus, 1.5 ng/50 mg of hindbrain and 1.5 ng/75 mg of optic tectum. After extraction, samples were transferred to clean centrifuge tubes, centrifuged (13,000 RCF, 10 min) and the supernatant collected. After addition of 1500 μ L of ultra pure water and 36 μ L of phosphoric acid, the samples were purified using the Strata-X-C SPE plates. Sorbents were conditioned with 1 mL methanol, equilibrated with 1 mL of 2% phosphoric acid in HPLC grade water prior to loading brain extracts onto the SPE wells. After a washing step using 1 mL HPLC grade water, sorbents were dried under vacuum for 15 min. Analytes were eluted with 1 mL of 5% ammonium hydroxide in methanol. Eluates were evaporated to dryness, reconstituted in 50 μ L of 80/20 water/methanol (v/v) and stored at -20 °C until analysis.

Plasma concentrations of the pharmaceuticals were measured for female fish where more volume of blood sample was available for chemical analyses. Plasma samples (150 μ L) were spiked with 1 ng of the mix of IS and 300 μ L of methanol and the mixture vortexed (1 min) and centrifuged (13,000 RCF, 10 min). The supernatant was collected, 1500 μ L of HPLC water and 36 μ L of phosphoric acid were added and samples were purified using the Strata-X-C plates as described previously, reconstituted in 50 μ L of 80/20 water/methanol (v/v) and stored at -20 °C until analysis.

2.2.2 Wastewater effluent

For each sample, one litre of wastewater containing 5% of methanol and 1% of acid acetic and spiked with the mix of IS was pre-filtered through glass wool and filter paper Whatman No1 (Whatman, Maidstone, UK). Samples were then extracted through an Oasis HLB (20 mL, 1 g) cartridge, which was pre-conditioned with 10 mL of methanol and 10 mL HPLC water. The cartridge was washed with 20 mL of distilled water and dried under vacuum. Analytes were eluted with 10 mL of methanol and 10 mL of ethyl acetate. Extracts were dried and reconstituted in 1 mL of methanol. 300 mL of the residue was purified using Strata-X-C plates described above and reconstituted to a final volume of 120 μ L (80/20 water/methanol (v/v)) and stored at -20 °C until analysis.

2.3 UHPLC-ESI-MS/MS

UHPLC-MS/MS analyses were carried out using a Waters Acquity UHPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Samples were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 μ m, 2.1 mm \times 100 mm, Waters, Manchester, UK) fitted with a ACQUITY UHPLC BEH C18 VanGuard pre-column (130 Å, 1.7 μ m, 2.1 mm \times 5 mm, Waters, Manchester, UK) and maintained at 22 °C. Injection volume was 20 μ L and mobile phase solvents were 94.8% water, 5% ACN, 0.2% formic acid (A) and 99.8% ACN, 0.2% formic acid (B). Separation was achieved using a flow rate of 0.1 mL/min with the following A:B gradient; 100:0 to 64:36 in 6 min; from 64:36 to 20:80 at 15 min, from 20:80 to 100% B at 15.01 min and held for 5 min prior to return to initial conditions and equilibration for 5 min.

MS/MS was performed in the multiple reaction monitoring (MRM) using ESI in the positive mode. Two characteristic fragmentations of the protonated molecular ion $[M+H]^+$ were monitored (one for quantification and the other one for confirmation). The declustering

potential and collision energy were optimised for each analyte (Table S1). Other parameters were optimised as follows: capillary voltage -3.3 kV, extractor voltage 8 V, multiplier voltage 650 V, source temperature 100 °C, desolvation temperature 300 °C. Argon was used as collision gas (P collision cell: 3×10^{-3} mbar), and nitrogen was used as desolvation gas (600 L/h). Data were acquired using MassLynx 4.1 and the quantification was carried out by calculating the response factor of neurotransmitters and pharmaceuticals to their respective IS. Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio of the analyte to the IS. In order to account for interferences with the matrix, calibration curves for the pharmaceuticals present in brain tissues and plasma analyses were made in the sample matrix using plasma and brain tissues collected from additional unexposed fish. A minimum of a six point calibration curve ($R^2 > 0.99$) was used to cover the range of concentrations observed in the different matrices, within the linear range of the instrument. Calibration curves were prepared in solvent only to quantify neurotransmitters as these were naturally occurring at high concentrations in all brain tissues and not subject to matrix interference. Method detection and quantification limits (MDL and MQL, respectively) were determined from spiked samples which had been extracted using the Strata-X-C method. MDLs were determined as the minimum amount of analyte detected with a signal-to-noise ratio of 3 and MQLs as the minimum amount of analyte detected with a signal-to-noise ratio of 10 (Table S2).

2.4 Plasma and brain bioconcentration factors

For the purpose of our study, we defined plasma and brain bioconcentration factors (BCFs) of pharmaceuticals as the ratio of blood plasma or brain to water concentrations for the specified exposure time adopted (i.e. 15 days) for which we would expect equilibrium saturation to have been reached, based on studies in other freshwater fish such as Japanese medaka and channel catfish where equilibrium concentrations for fluoxetine (Paterson and

Metcalfe, 2008) and for diazepam (Overturf et al., 2016) were reached within 2-3 days of exposure. BCFs were calculated as follows:

$$BCF = C_{\text{plasma or brain}} / C_{\text{ambient water}}$$

Where $C_{\text{plasma or brain}}$ and $C_{\text{ambient water}}$ are the chemical concentrations in plasma or brain of fish and in ambient water (i.e., the WwTW effluent), respectively. C_{plasma} and $C_{\text{ambient water}}$ were both expressed in ng/mL while C_{brain} was expressed in ng/g (wet weight). $C_{\text{ambient water}}$ was calculated from the mean concentration of the 3 sampling time points (day 0, 7 and 15 days of the fish exposure period) and C_{plasma} and C_{brain} were calculated from the average concentrations measured in plasma and the four parts of the brain respectively. To calculate the average concentrations measured in the four parts of the brain of the effluent-exposed fish, the total amount found in the four parts of the brain was first calculated and then divided by the total mass of brain tissues analysed for each individual fish.

Predicted BCF_{plasma} , were calculated using fish steady-state plasma concentration (FssPC) which can be predicted using the Fish Plasma Model as follows (Huggett et al., 2003):

$$FssPC = [\text{water concentration, ng/mL}] \times P_{b/w}$$

$P_{b/w}$ corresponds to blood to water partition coefficient and can be calculated using the following equation:

$$\text{Log } P_{b/w} = 0.73 \times \text{Log } D_{7.4} - 0.88$$

In the last equation, $\text{Log } D_{7.4}$ (i.e., $\text{Log } D$ value at buffer pH of 7.4) was used instead of $\text{Log } K_{ow}$ as several studies have shown that the ambient pH significantly affects the ionization of the pharmaceuticals analysed in this study (Margiotta-Casaluci et al., 2014; Nallani et al., 2016; Tanoue et al., 2015; Valenti et al., 2009). $\text{Log } D_{7.4}$ were obtained from chemspider (<http://www.chemspider.com/>).

2.5 Quality control

One workup sample (i.e. extraction using extraction methods without a tissue/plasma sample) per batch of 12 samples was injected on the UHPLC-MS/MS at the beginning of the run to ensure that no contamination occurred during the sample preparation. Solvent samples (methanol:water, 20:80) were also injected between sample batches to rule out the possibility of any carryover in the UHPLC system that might affect adjacent results in analytical runs. Identities of detected neurotransmitters and pharmaceuticals were confirmed by comparing ratios of MRM transitions in samples with that of pure standards. Quality control samples (QCs, i.e., standard solutions) were injected before all sample batches to monitor sensitivity changes and every 12 samples to monitor the sensitivity changes during the analysis of each batch. Recovery experiments performed on spiked brain tissues (50 ng/g, n=4), plasma samples (10 ng/mL, n=4) and HPLC water (8.5 ng/mL, n=4) gave absolute recovery values ranging from 61 ± 7 to $107 \pm 14\%$ for neurotransmitters and pharmaceuticals (Table S3). None of the studied pharmaceuticals were detected in the samples from control fish.

2.6 Statistical analysis

All statistical analyses were carried out using GraphPad Prism 5 software. To compare pharmaceutical and neurotransmitter concentrations in males and females between treatments, 12 fish per treatment were analysed by taking randomly 4 fish (2 males and 2 females) from each triplicate tank (n=3 for biological replicates per treatment and n=12 for analytical replicates). Data was tested for normality using the Shapiro-Wilk test. Differences in pharmaceutical concentrations between effluent-exposed males and females in different parts of the brain were assessed by two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison tests. One-way ANOVA followed by Bonferroni multiple comparison tests were used to compare pharmaceutical concentrations in the different parts of

the brain of effluent-exposed male or female fish. Nonparametric Mann–Whitney (M–W) U tests were used to compare neurotransmitter concentrations between control and exposed males and females. Differences were considered statistically significant at $p \leq 0.05$. Statistical results for the different tests, including the value of the test, the degrees of freedom, and the p-values are provided in Table S5 (two-way ANOVA), S6 (one-way ANOVA, male), S7 (one-way ANOVA, female) and S9 (Mann–Whitney U-tests). To perform the statistical analyses, all concentrations that were over the limits of detection (\geq MDL) but below the limits of quantification ($<$ MQL) were assigned the value considered as the MDL in each case. Concentrations below the MDL were considered to be zero.

3. Results and Discussion.

3.1. Concentrations of neuroactive pharmaceuticals in wastewater of effluent-exposed roach after 15 days of exposure.

Thirteen of the 14 test pharmaceuticals (norclozapine excluded) were detected in the WwTW effluent sampled during the exposure period (Table 1). Mean concentrations ranged from 8.5 ± 1.1 ng/L (paroxetine) to 374 ± 44 ng/L (amitriptyline). Individual pharmaceutical concentrations measured in the WwTW effluent during the exposure period were in the same range as that reported in other WwTW effluents in the UK (i.e., between 10 and 500 ng/L) (Petrie et al., 2015). For example, mean concentrations of fluoxetine in our study were 62 ± 5.6 ng/L compared with a median of 23 ng/L and 95 percentile of 69 ng/L for fluoxetine measured in a comprehensive study of 162 WwTW effluents in the UK (Gardner et al., 2012). Likewise, mean concentrations of norfluoxetine (37 ± 13 ng/L), venlafaxine (356 ± 39 ng/L) and amitriptyline (374 ± 44 ng/L) in our study aligned with those measured in several other UK WwTW effluents (Petrie et al., 2015) indicating that our study effluent

content of neuroactive pharmaceuticals was representative of UK WwTW effluents more widely.

The detection of mixtures of SSRIs and other neuroactive compounds in WwTW effluents is of potential environmental concern because they have the potential to alter fish behaviour (Brodin et al., 2013; Margiotta-Casaluci et al., 2014; Valenti et al., 2009). However, concentrations of the SSRIs fluoxetine, sertraline and citalopram and the SNRI venlafaxine measured in the undiluted effluent in our study were well below nominal concentrations that have been shown to affect fish behaviour under experimental laboratory conditions. For instance, exposure to water concentrations of sertraline at $> 3 \mu\text{g/L}$ were necessary to cause changes in shelter-seeking behaviour in adult male fathead minnows (Valenti et al., 2012) and shoaling activity in crucian carp (Xie et al., 2015). Similarly, other studies investigating the effects of exposure to fluoxetine, citalopram or venlafaxine have shown that behaviour-related changes in fish were only observed after exposure to concentrations in the $\mu\text{g/L}$ range (Bisesi et al., 2014; Kellner et al., 2016; Margiotta-Casaluci et al., 2014). In contrast to a number of studies investigating exposure effects of SSRIs on fish behaviour, little is known on the potential behavioural effects of exposure to atypical antipsychotics such as clozapine or tricyclic antidepressants such as amitriptyline. In addition, risk assessment studies are based on exposures to single chemicals and do not reflect the complex and diverse mixtures of neuroactive pharmaceuticals that fish are exposed to in WwTW contaminated waters as revealed in our study.

316 **Table 1.** Pharmaceutical concentrations in wastewater effluent and bioconcentration factors (BCFs) in effluent-exposed fish.

| | [Effluent] | | [Fish plasma] | | BCF _{plasma} | | BCF _{brain} | [Brain]/ [Plasma] |
|----------------------------------|--------------------|-------------------|---|--------------------|-----------------------|-----------|----------------------|----------------------|
| Pharmaceuticals | Range (ng/L) | Av ± SD (ng/L) | Range (ng/mL) | Av ± SD (ng/mL) | measured | predicted | measured | |
| SSRIs | | | | | | | | |
| fluoxetine | 54 - 72 | 62 ± 5.6 | 0.33 - 0.45 | 0.39 ± 0.04 | 6.3 | 2.5 | 68 | 9.7 |
| norfluoxetine | 17 - 61 | 37 ± 13 | 0.43 - 0.83 | 0.53 ± 0.15 | 14 | 5.6 | 285 | 18 |
| sertraline | 47 - 65 | 55 ± 5.6 | 0.5 - 1 | 0.76 ± 0.21 | 14 | 26 | 361 | 27 |
| norsertaline | 215 - 311 | 260 ± 31 | 3.3 - 15 | 9.5 ± 4.7 | 37 | 15 | 1581 | 37 |
| citalopram | 211 - 340 | 274 ± 39 | <0.02 ^a - <0.05 ^b | n.a. | n.a. | n.a. | 1.6 | n.a. |
| paroxetine | 6.6 - 9.8 | 8.5 ± 1.1 | <0.17 ^a | n.a. | n.a. | n.a. | 67 | n.a. |
| Atypical antipsychotic | | | | | | | | |
| clozapine | 20 - 30 | 25 ± 3.5 | <0.07 ^a - <0.2 ^b | n.a. | n.a. | n.a. | 105 | n.a. |
| norclozapine | <0.08 ^a | | <0.33 ^a | n.a. | n.a. | n.a. | n.a. | n.a. |
| quetiapine | 18 - 33 | 23 ± 6.2 | <0.03 ^a - <0.1 ^b | n.a. | n.a. | n.a. | 157 | n.a. |
| Tricyclic antidepressants | | | | | | | | |
| amitriptyline | 298 - 421 | 374 ± 44 | 0.90 - 2.2 | 1.5 ± 0.5 | 4.0 | 19 | 15 | 4.4 |
| noramitriptyline | 31 - 49 | 43 ± 7.6 | <0.20 ^b - 0.3 | 0.16 ± 0.1 | 3.7 | 6.1 | 42 | 14 |
| SNRI | | | | | | | | |
| venlafaxine | 295 - 393 | 356 ± 39 | 0.03 - 0.1 | 0.07 ± 0.02 | 0.2 | 1.5 | 0.7 | 3.7 |
| Benzodiazepine | | | | | | | | |
| diazepam | 10 - 26 | 19 ± 6.2 | <0.02 ^a | n.a. | n.a. | n.a. | n.a. | n.a. |
| nordiazepam | 56 - 96 | 77 ± 15 | <0.50 ^b - 0.55 | 0.51 ± 0.01 | 6.6 | 9.3 | 2 | n.a. |

317 Wastewater effluents were sampled at the beginning of the experiment, after 7 days and the end of the experiment. Samples were analysed in
318 triplicates for each sampling point (n=9 in total). BCF_{brain} was calculated from the sum of concentrations measured in the brain regions. ^a =
319 MDL values; ^b = MQL values, SD= standard deviation and n.a. not applicable as concentrations in effluent, plasma or brain extracts were <
320 MQL. For mean calculations, concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were
321 assigned the MDL value. Concentrations below the MDL were considered to be zero.

3.2. Concentrations of neuroactive pharmaceuticals in plasma and brain of effluent-exposed roach

After a 15 day exposure to WwTW effluent, 11 of the 14 test compounds were detected in plasma, the exceptions being paroxetine, norclozapine and diazepam. No analytes were detected in plasma from control fish (Table 1). Citalopram, clozapine and quetiapine and noramitriptyline were present at concentrations below the MQL. Mean measured concentrations of all other analytes were between 0.07 ± 0.02 (venlafaxine) and 9.5 ± 4.7 ng/mL (norsertaline). For the analytes where plasma concentrations were above the MQL, the measured BCF_{plasma} ranged from 0.2 to 37, in agreement with the BCF_{plasma} values for SSRIs and venlafaxine from a previous exposure study in which rainbow trout were exposed to an undiluted (Swedish) WwTW effluent for 13 days (Grabicova et al., 2014). In our study, the measured BCFs were in the same order of magnitude but differed by between 2- to 7 -fold lower or higher compared to their predicted BCF_{plasma} values (Table 1). A previous study (Margiotto-Casaluci et al., 2014) has reported an inter-individual variability up to 7.6-fold in drug uptake (water/plasma), which would suggest that the differences between measured and predicted BCFs in our study are within an acceptable level of accuracy. Possible reasons for the discrepancies between measured and predicted BCF_{plasma} values for these pharmaceuticals, as well as for other xenobiotics, may involve water pH (Valenti et al., 2009), routes of exposure other than via diffusion through the gills (e.g., the diet), the effects of uptake/elimination mechanisms at gill membranes, plasma protein binding, and/or hepatic clearance rates (Tanoue et al., 2015).

Analysis of brain tissues of roach after a 15 day exposure to WwTW effluent revealed that 13 neuroactive pharmaceuticals were detected in the optic tectum, 11 in the hindbrain and 10 in both the telencephalon and the hypothalamus (Figure 1, Table S4). None of the

pharmaceuticals were detected in brain regions of control fish. Diazepam was not detected in any of the brain tissues of effluent-exposed fish and its biologically active metabolite nordiazepam was only detected in the optic tectum but at concentrations below the MQL. Both diazepam and nordiazepam are rapidly metabolized in vertebrate tissues (Friedman et al., 1986) which may account for the low levels present in brain and plasma despite being present at a concentration of 19 ± 6 ng/L (mean \pm SD) in the effluent. The highest concentrations of pharmaceuticals detected in brain tissues of male and female roach were norsertraline (459 ± 89 ng/g in the hindbrain), followed by sertraline (21 ± 6.9 ng/g in the optic tectum), norfluoxetine (14 ± 2.5 ng/g in the telencephalon), norclozapine (9.4 ± 5.8 ng/g in the hindbrain), amitriptyline (7.5 ± 2.7 ng/g in the hindbrain), and fluoxetine (5.7 ± 4 ng/g in the hindbrain) (Table S4). Mean concentrations of citalopram, paroxetine, venlafaxine and nordiazepam were ≤ 1 ng/g in all brain tissues. Paroxetine and nordiazepam were detected only in the optic tectum and not in other brain tissues. This was most likely because the greater mass of tissue available for the analysis of the optic tectum that was on average 1.4, 4.4 and 5.3 times higher than for the hindbrain, the telencephalon and the hypothalamus, respectively, thus influencing the MDL. Likewise, the MDL for norclozapine was higher than that of the other compounds (Table S2) which may explain why it was only detected in the optic tectum and the hindbrain.

These findings are in agreement with reports that fluoxetine, sertraline and their “nor” metabolites are common SSRIs detected in the brain of fish species collected in an effluent-dominated stream in USA (Brooks et al., 2005), in the brain of carp collected in an effluent-dominated river in Japan (Tanoue et al., 2015) and in the brain of brook rainbow exposed to a diluted effluent from a WwTW in Canada (Lajeunesse et al., 2011). Sertraline, as well as citalopram and venlafaxine were also detected in the brain of rainbow trout exposed to an undiluted WwTW effluent in Sweden (Grabicova et al., 2014) and the antidepressant

bupropion and the antipsychotic risperidone have been found to accumulate in the brain of round goby exposed to undiluted WwTW effluents in Canada (McCallum et al., 2017). Here we show that other neuroactive drugs including the antipsychotics clozapine, norclozapine and quetiapine as well as the tricyclic antidepressants amitriptyline and its 'nor' metabolite also bioaccumulate in fish brain together with SSRIs and venlafaxine.

Accumulation of pharmaceuticals in the brain of effluent-exposed fish was not affected by sex except for nortriptyline which was present at slightly higher concentrations (1.2-1.7-fold) in males compared with females in the telencephalon, hypothalamus and optic tectum ($p < 0.05$) but not in the hindbrain (Figure 1). Generally, profiles of the pharmaceuticals were similar between the different regions of the brain of effluent exposed fish. Direct comparison of the distribution of sertraline, nortriptyline, norfluoxetine and quetiapine between brain regions was possible as they were present at concentrations above the MQL in all four brain regions. However, quetiapine concentrations in both sexes were significantly higher in the optic tectum, sertraline and nortriptyline were generally lower in the telencephalon and norfluoxetine (females only) significantly higher in the telencephalon compared with other brain regions ($p < 0.05$, See Figures S1 and S2). The relatively uniform concentrations of SSRIs in different regions of the human brain after autopsy has been reported previously indicating that there are little regional differences in their uptake and metabolism between brain compartments (Wille et al., 2009). The ratios of concentrations of the demethylated 'nor' metabolites to the parent compound were measured for fluoxetine, sertraline and amitriptyline in the effluent samples and were close to the values reported in human plasma (Table S8). However, the same ratios measured in fish plasma and brain regions were consistently higher for fluoxetine and sertraline compared with the effluent indicating further metabolism to the 'nor' metabolite within the fish tissues.

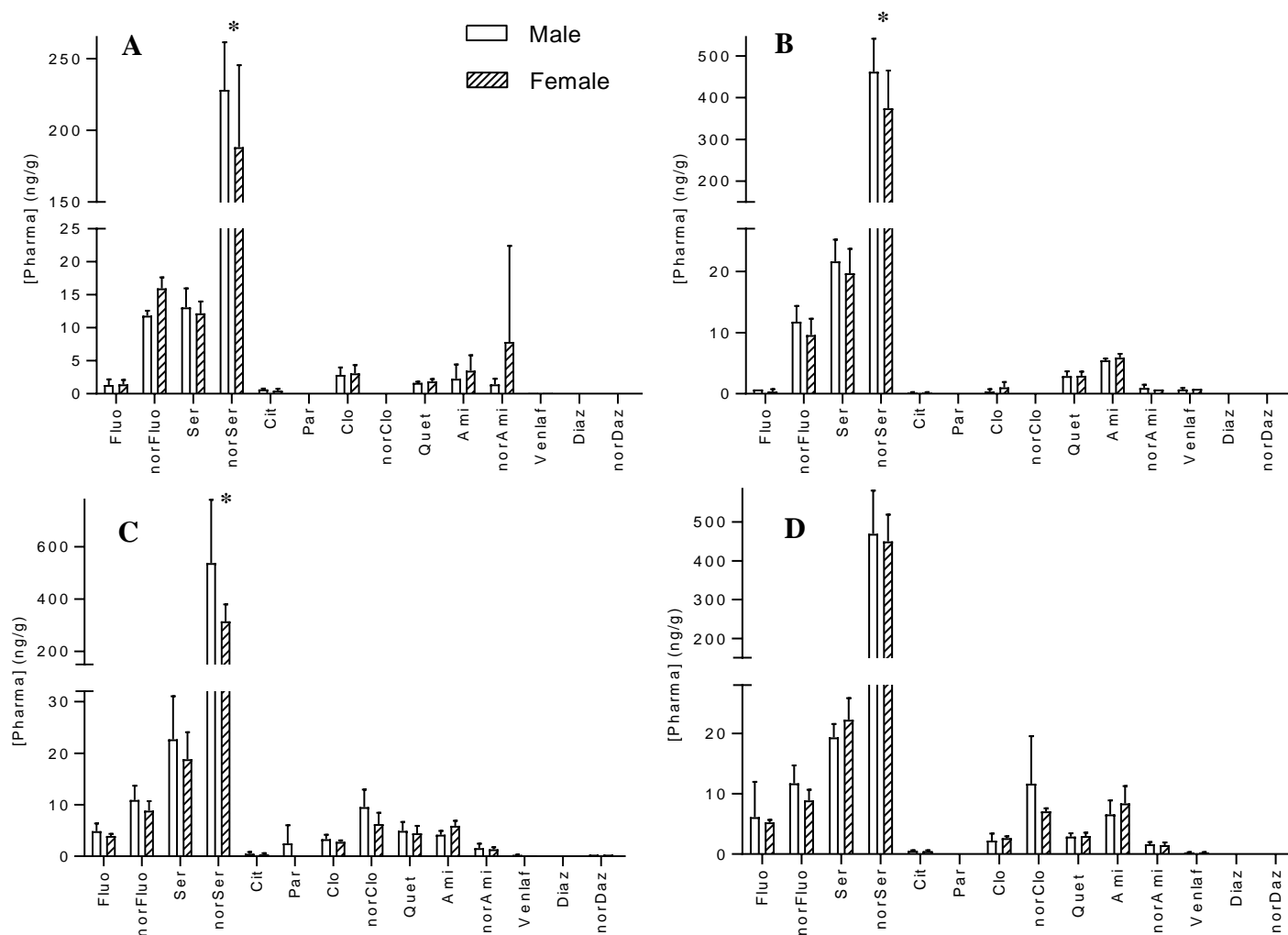


Figure 1. Concentrations of neuroactive pharmaceuticals (ng/g; mean \pm standard error) in the telencephalon (A), hypothalamus (B), optic tectum (C) and hindbrain (D) of effluent-exposed male and female fish. Brain tissue from 12 fish (6 males and 6 females) sampled from three replicate tanks (2 males and 2 females per tank) were analysed. None of the studied pharmaceuticals were detected in the control fish. * significant difference between male and female fish $p \leq 0.05$. Fluo= fluoxetine, norFluo= norfluoxetine, sertraline, norSer= norsesertraline, Cit= citalopram,

415 Par= paroxetine, Clo= clozapine, norClo=norclozapine, Quet= quetiapine, Ami=amitriptyline, norAmi= noramitriptyline, Venlaf= venlafaxine,
416 Diaz= diazepam, norDaz= nordiazepam.

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The measured BCF_{brain} values of pharmaceuticals in the summed brain regions were 3-40 fold higher than measured $BCFs_{\text{plasma}}$ and ranged from 0.7 for venlafaxine to 1581 for nortriptyline, indicating efficient transport and uptake into brain tissues. Similar BCF_{brain} values and brain/plasma concentration ratios of SSRIs have been reported in fish exposed to WWTW effluents (Grabicova et al., 2014; Lajeunesse et al., 2011; Tanoue et al., 2015). In our study, compounds such as paroxetine, clozapine and quetiapine did not bioconcentrate in the plasma ($BCFs < 1$), but these compounds were quantified at higher concentrations in the brain with $BCFs$ between 67 and 157. Our data emphasise the importance of analysing target tissues of pharmaceuticals where there is a greater potential for bioaccumulation compared with the fish blood plasma.

3.3. The effect ratio (ER).

Comparison of fish plasma levels with human therapeutic plasma concentrations (HtPCs) has been proposed as a technique to estimate the potential hazards of pharmaceuticals in wild fish (Huerta et al., 2016; Margiotta-Casaluci et al., 2014; Tanoue et al., 2015). The read-across hypothesis has been proposed as a first step to study the potential for adverse ecotoxicological effects due to exposure to environmental pharmaceuticals and remains to be proven for most biologically active compounds (Huggett et al., 2003; Rand-Weaver et al., 2013). The ER was calculated from a range of HtPCs measured in human samples (Regenthal et al., 1999; Schulz et al., 2012) and the measured or predicted fish plasma concentrations of pharmaceuticals (Table 2). An $ER \leq 1$ indicates that the predicted drug concentration in the fish plasma is equal to or greater than the drug concentration in human plasma that elicits a therapeutic effect. Measured ER values (i.e., based on measured plasma concentrations) were generally similar to predicted values and ranged from 33-5714 for all compounds, and were between 39-1282 for SSRIs. In addition, combined ERs were

calculated for the 3 SSRIs sertraline, fluoxetine and norfluoxetine by assuming an additive effect. Sertraline being the most potent, was used as the reference and relative potencies of fluoxetine and norfluoxetine to sertraline were calculated based on respective HtPCs. The calculated combined ER values for these 3 compounds ranged from 31 to 195. Norsertraline was not considered in this mixture as it has only 5–10% of the serotonin reuptake inhibitor potency of sertraline and therefore a minimal contribution to clinical effects of sertraline (Hiemke and Hartter, 2000). Based on the HtPC values, and the assumptions of the read-across hypothesis, then the ER data in Table 2 would suggest that none of the pharmaceuticals would have a therapeutic effect in fish. However, it should be noted that the read-across hypothesis still has to be validated for many of the SSRIs and other neuroactive pharmaceuticals investigated in this study; that knowledge of the behavioural and other endpoints of their action in fish remain incomplete; and the effects of SSRI exposure in concert with mixtures of other neuroactive substances remains uncertain. A safety factor of 1000 for ER has been proposed to take into consideration extrapolation of humans to animals, sensitivity differences, and extrapolation from mammalian to non-mammalian species (Huggett et al., 2003). The ER ranges of fluoxetine, sertraline, norfluoxetine and amitriptyline fall within this safety factor indicating that these compounds in fish should be prioritised for risk assessment studies, especially if additive effects can be expected between SSRIs. However, it should also be noted that the lower bound of ER ranges for compound such as noramitriptyline, venlafaxine and nordiazepam were below 1000, and therefore effects cannot be totally excluded for these compounds if the same HtPC values are applicable to fish.

Table 2. Human therapeutic plasma concentrations (HtPC) and effect ratio of analysed pharmaceuticals

| | HtPC ¹ | Effect ratio fish plasma ² | |
|----------------------------------|-------------------|---------------------------------------|------------|
| Pharmaceuticals | ng/mL | measured | predicted |
| SSRIs | | | |
| fluoxetine | 100 -500 | 256 - 1282 | 646 - 3229 |
| norfluoxetine | 150 - 400 | 283 - 755 | 724 - 1932 |
| sertraline | 30 -250 | 39 - 329 | 21 - 176 |
| norsertraline | unknown | - | - |
| citalopram | 10 - 200 | >500 - 10000 ^a | 32 - 645 |
| paroxetine | 10 - 70 | nd | - |
| Atypical antipsychotic | | | |
| clozapine | 300 - 800 | >4286 - 10000 ^a | 938 - 2500 |
| norclozapine | 100 - 300 | nd | - |
| quetiapine | 20 – 500 | >667 - 16667 ^a | 143 - 3571 |
| Tricyclic antidepressants | | | |
| amitriptyline | 50 – 300 | 33 - 200 | 7 - 42 |
| noramitriptyline | 60 – 300 | 375 - 1875 | 231 - 1154 |
| SNRI | | | |
| venlafaxine | 30 – 400 | 429 - 5714 | 58 - 770 |
| Benzodiazepine | | | |
| diazepam | 200 - 2000 | nd | - |
| nordiazepam | 20 – 800 | 39 - 1569 | 28 - 1121 |

¹ HtPC were obtained from Regenthal et al. (1999) and Schulz et al (2012).

²Effect ratio was calculated as HtPC/C_{plasma}, where C_{plasma} in fish was an average of the measured values in the plasma or the predicted values from mean concentrations measured in the ambient wastewater effluent. The range of effect ratios are based on the range of published HtPC values.

^a based on C_{plasma} MDL value when plasma concentrations were between MDL and MQL. nd = not detected (i.e. concentrations <MDL).

3.4 Neurotransmitter concentrations in the brain of control and effluent-exposed fish

The concentrations of 4 neurotransmitters (serotonin, tryptophan, acetylcholine and glutamate) were measured in the four brain regions of male and female roach to investigate differences in brain chemistry between control and effluent-exposed fish. Serotonin and its precursor tryptophan were selected since SSRIs act by inhibiting the serotonin transporter in the pre-synaptic cell, which in turn leads to increased extracellular levels of serotonin in the synaptic cleft (Hiemke and Hartter, 2000). Likewise, the SNRI venlafaxine and the tricyclic antidepressants act by blocking the serotonin transporter and could impact on serotonin

concentrations in the brain. Tricyclic antidepressants also have high affinity for antagonising the muscarinic acetylcholine receptors and therefore act as potent anticholinergics (Snyder and Yamamura, 1977). Glutamate was also analysed as it is a precursor of gamma-aminobutyric acid (GABA) and benzodiazepines are designed to alter behaviour by binding to GABA receptors (Brodin et al., 2013).

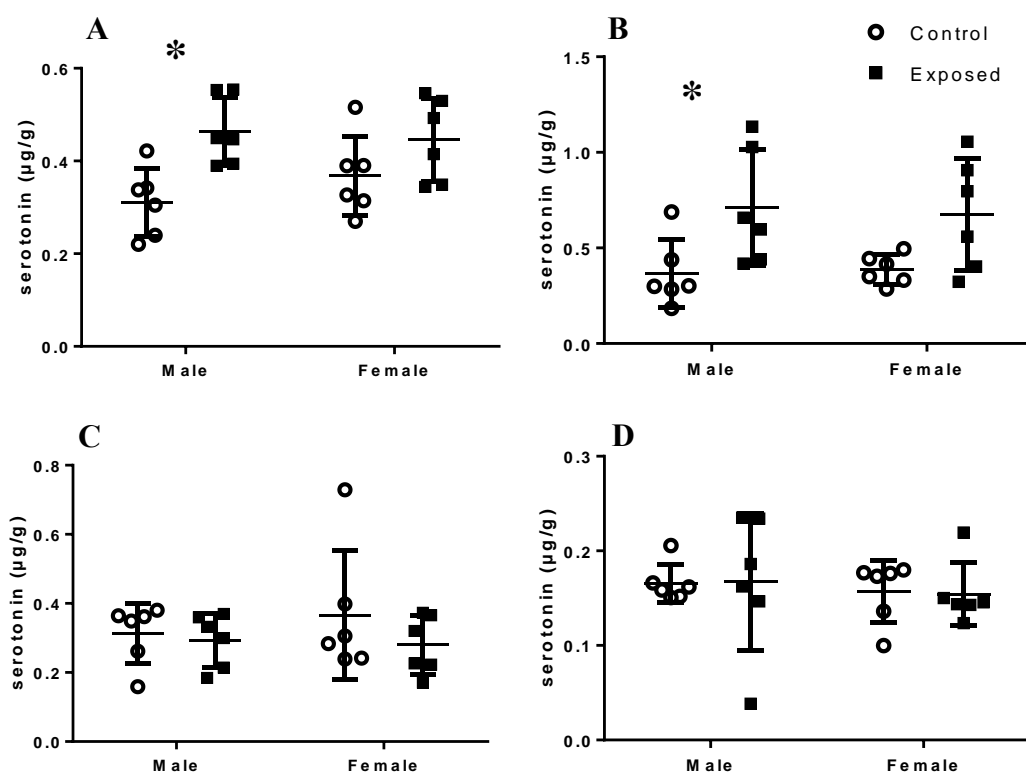
The most abundant neurotransmitter found in the brain tissues of control or effluent exposed fish was glutamate (between 2.8-259 $\mu\text{g/g}$), followed by tryptophan (3.2-57 $\mu\text{g/g}$), acetylcholine (0.22-6.3 $\mu\text{g/g}$) and serotonin (0.04-1.1 $\mu\text{g/g}$). The concentrations of neurotransmitters detected in the brain tissues of control or effluent-exposed fish were present at similar levels to that reported in the mammalian brain (Gonzalez et al., 2011). Serotonin concentrations were also similar with those reported for different regions of the brain in rainbow trout (Sloman et al., 2005). Small but significant increases in serotonin concentrations were observed in the hypothalamus (1.9 fold, $p<0.05$) and the telencephalon (1.5 fold, $p<0.01$) in effluent-exposed male but not female roach (Figure 2). A significant decrease in glutamate (4 fold, $p<0.05$) and acetylcholine concentrations (2.8 fold, $p<0.05$) was observed for the effluent-exposed females in the hypothalamus and a significant glutamate increase (2.0-3.1 fold, $p<0.05$) for the effluent-exposed males and female in the hindbrain (Figures S3 and S4). A significant increase in tryptophan concentrations were also observed for the effluent-exposed males in the hypothalamus (1.7 fold, $p<0.05$) and the optic tectum (1.5 fold, $p<0.05$) (Figure S5). Our data suggests that the disruption of neurotransmitter concentrations in the brain of effluent-exposed fish is sex dependent. However, an examination of the data in Figures 2 and S3-S5 reveals that the trend in responses is similar in both sexes, and further work with increased replication is needed to determine whether there are gender specific responses to effluent exposure. It is not known whether the disruption of neurotransmitter concentrations observed in our study would lead to

changes in fish behaviour and further studies would be required to determine this. Monoaminergic systems play a crucial role in linking behaviour and physiology in fish, and brain serotonin levels can influence food intake, cognitive ability, locomotor activity and social behaviour in fish (Bisesi et al., 2014; Sloman et al., 2005; Winberg et al., 1996). In addition, serotonergic modulation in the hypothalamus similar to that observed in this study has the potential to affect sex hormones and modulate genes involved in reproductive function and behaviour in the brain of female goldfish (Mennigen et al., 2008; Prasad et al., 2015).

Further work is required to ascertain whether disruption of neurotransmitter levels caused by effluent exposure is due solely to exposure to the pharmaceutical mixtures analysed in this study or a result of combined exposure to other contaminants such as neurotoxic insecticides which could potentially be present in the effluent and contribute to the alteration of fish neurotransmitter levels (Tufi et al., 2016). Furthermore, our study was based on exposure to an undiluted treated effluent, which may reflect the exposure conditions of aquatic organisms in some countries where there is little or no dilution of the discharges into the receiving waters during the summer period (David et al., 2013), but would need to be conducted on diluted effluents to be representative of most ambient river environments.

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539

540 **Figure 2.** Serotonin concentrations (µg/g) in the telencephalon (A), hypothalamus (B), optic
 541 tectum (C) and hindbrain (D) of control and effluent-exposed male and female fish (n=6). *
 542 $p \leq 0.05$. Each dot represents an individual fish within the treatment group, alongside the mean
 543 \pm standard error of the replicates.
 544

545 Conclusions

546 This study reports for the first time that a highly complex mixture of 13 neuroactive
 547 pharmaceuticals, comprising SSRIs, SNRIs, atypical antipsychotics, tricyclic antidepressants
 548 and benzodiazepines, accumulates in multiple brain regions of fish exposed to a treated
 549 WWTW effluent. Concentrations of all pharmaceuticals were below their respective HTPCs
 550 however, disruptions in serotonin, glutamate and acetylcholine concentrations were observed
 551 in different brain regions of effluent-exposed fish. Future risk assessment studies should be
 552 based on understanding the effects of exposure to these complex chemical mixtures on fish
 553 behaviour and physiology, particularly as future anthropogenic pressures are likely to result

in increased pharmaceutical exposures and fluctuating hydrological conditions in river waters.

Supporting Information. Supporting information comprises UHPLC-MS/MS acquisition parameters; MDL, MQL and method recoveries for neurotransmitter and pharmaceutical compounds in extracts of brain tissues, plasma and water and additional tables and figures with pharmaceutical and neurotransmitter concentrations in WwTW effluents, plasma and brain tissues.

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